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The Utilization of Acrylic Polymers for Separation of Biological Materials and as a Matrix for Enzymes

Lund 1972

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This thesis is based on the following papers.

I. Hjertén, St. and Mosbach, R.: "Molecular-Sieve" Chromatography of Proteins on Columns of Cross-linked Polyacrylamide. Anal. Bioch., 3, 109 (1962).

II. Mosbach, R.: Chromatographic Separation of Substances of Different Molecular Weight. U.S. Patent No. 3, 298, 925 (1967).

III. Mosbach, K. and Mosbach, R.: Entrapment of Enzymes and Microorganisms in Synthetic Polymers and their Application in Column Techniques. Acta Chem. Scand., 20, 2807 (1966).

IV. Nilsson, H., Mosbach, R. and Mosbach, K.: Preparation of immobilized enzymes through bead-polymerization of acrylic monomers. Biochim. Biophys. Acta, 268, 253 (1972).

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SURVEY

The general theme of this thesis is the utilization of acrylic polymers for two purposes.

In Part A, covering publications (I=2) and (II=3), the separation of biological materials with special reference to chromatography is discussed

The successful application of chromatography (gel filtration) with cross-linked dextrans as matrix for distributing solutes differeing in size stimulated the development of other gels to be used likewise. An alternative gel was developed based mainly on acrylic monomers. In particular, cross-linked polyacrylamide has been found useful since gels of specific pore-size can be made with great ease, thus permitting preparation of "tailor-made gels". Ionic gels also can be made by copolymerization with monomers or through subsequent introduction of ionic groups into the polymer chain formed. In publication (I=2) examples are given that demonstrate the separation potential of columns packed with particles of cross-linked polyacrylamide (acrylamide and N,N´metylene bis (acrylamide)) using a mixture of R-phycoerythrin, R-phycocyanin, hemoglobin, cytochrome c, DNP-aspartic acid and naphthol green. Publication (II=3) deals primarily with the preparation of a variety of acrylic polymers and other compounds of vinylic types.

In Part B, covering publications (III=4) and (IV=6), the application of acrylic polymers as matrices for immobilized enzymes and microorganisms is discussed

The binding of enzymes to such supports permits repeated use of these bound biocatalysts. Stabilization of the enzymes may also be attained. Acrylic polymers show several of the properties required of a matrix to be used as support for enzymes, such as rigidness, hydrophilicity, and inertness to biological degradation.

Of the different binding techniques possible, the so-called entrapment method, leading to the entrapping of enzymes in the gel network of such

polymers, has been one of the first to be used (publication III=4) and is easily carried out. A further improvement of this technique leading to well-defined spherical particles of desired diameter containing the included enzyme is described (publication IV=6). The bead-polymerization technique described here represents an improvement of the original polymerization procedure, the latter leading to a gel-bulk requiring subsequent granulation. One advantage inherent in such preparations is their possible use in enzyme therapy. Further, they show good flow rates when used in column processes and constitute valuable models för kinetic studies.

INTRODUCTION

In the beginning of the 1950:s polyacrylamide and its derivatives had found application in various specific branches of industry where it is used as an adhesive, thickening agent, protective colloid, flocculant, soil stabilizer and for surface coating. Cross-linked polyacrylamide has also been used for electrophoresis as a stabilizing medium since 1956.

The development of cross-linked dextrans as a powerful tool in the separation of biological substances in 1959 (1) stimulated the search for other materials with similar properties to be used in chromatography.

In 1962 the preparation and application of cross-linked polyacrylamides were described (I=2, II=3), showing similar properties. Such preparations have subsequently, like the dextrans, found wide use in chromatography of biological material. A different with high potential area lies in the use of such gel material as a matrix or support for enzymes, as demonstrated in 1966 (III=4,5). Such immobilized enzyme preparations may find application in another chromatographic method, that of affinity chromatography or biospecific adsorption, e.g. the isolation of inhibitors and enzymes. They may also be used in enzyme technology, or as model systems for in vivo situations, where most of the enzymes are bound or embedded in membranous structures.

Later, in 1972, a more sophisticated development of the entrapping technique, leading to spherical particles of uniform size containing the entrapped enzyme, was developed (IV=6). These preparations show potential for enzyme therapy and other areas.

PART A

Acrylic polymers in separation

The search for suitable methods for the separation and fractionation of proteins, polysaccharides and nucleic acids in particular, has been a major goal in biochemistry. In this context it was important to obtain matrices for use in chromatography, giving separation without denaturation and irreversible sorption taking place. The introduction of calcium phosphate by Tiselius et al. (7) and of ion-exchangers, especially those of cellulose type, by Sober and Peterson (8), represented developments important for the general area of protein chemistry. With the above matrices, a multi-step elution was usually used to obtain separation. However, in doing so, even homogeneous substances may often give rise to several peaks, so-called "false components" in the chromatogram, because of the necessity of eluting stepwise with different concentrations of the eluant. Gradient elution was applied to protein chromatography to circumvent these difficulties. However, zones are often obtained showing non-linear distribution isotherms. The introduction by Porter (9) of liquid-liquid chromatography ("partition" chromatography) for proteins, using "kieselguhr" as support for the stationary liquid phase represented an important progress for obtaining the required "distribution" by the procedure of one-step development (elution chromatography). However, only a fair separation with the solvent system used was obtained.

Finally the introduction of hydrophilic gel particles, in particular of cross-linked dextrans for chromatography (gel filtration) by Porath and Flodin in 1959 (1), represented a breakthrough. This permitted group separation of high molecular weight substances, such as proteins, from low-molecular weight substances, and the fractionation of peptides and proteins (10,11). Other gels useful in chromatographic separations introduced were agar-agar (12). agarose (13) and cellulose (14).

In a parallel study, published in 1962 (I=2), cross-linked polyacrylamide was used for the fractionation of proteins showing behavior similar to that observed with the dextrans, i.e. distribution of solutes according to size ("molecular sieve" chromatography). In the study under discussion here, the separation of phycoerythrin (mol.wt. 290,000), R-phycocyanin (mol.wt. 135,000 at pH 7.5), human hemoglobin (mol.wt. 68,000) and cytochrome c from horse heart (mol.wt. 13,000) was achieved obtaining Rf-values of 0.90, 0.78. 0.64 and 0.40, respectively as determined using diluted india ink as reference.

These Rf-values correspond to the retention ratios $R = \frac{v_o}{v_e}$ (v_o = void volume, v_e = effluent volyme).

For an arbitrarily chosen stationary phase v_s, and a mobile phase v_o, the relation $R = \frac{v_o}{v_o + K \cdot v_s}$ is valid. (K, the distribution coefficient = concentration ratio stationary/mobile phase.) The relation was originally found while studying "partition" chromatography

Two parameters for the distribution coefficients have frequently been used: $K_D = \frac{v_e - v_o}{v_i}$ (v_i = imbibed volume) and $K_{av} = \frac{v_e - v_o}{v_x}$ (v_x = total gel volume). The observed correlation of R-value or distribution coefficient K with the molecular weight of the protein led us to the assumption that the distribution of solutes between stationary and mobile phases takes place according to the Brönsted formula $K = e^{-\frac{\lambda}{RT}}$ also in "molecular sieve" chromatography. The latter formula is derived from the Boltzmann distribution law (λ is proportional to the molecular dimension of the solute). The stationary phase is considered to be made up of both matrix and solvent as a whole. The equation -log K = constant x M was originally derived by Brönsted (15) for isochemical low-molecular weight substances or homologues for liquid two-phase systems. A similar correlation valid in "partition" chromatography, has been pointed out by Martin (16). Albertson (17) developed an equation for the partition of spherical macromolecules in liquid two-phase systems based on the interfacial tension concept, substituting the molecular weight by the surface area $A \approx M^{\frac{2}{3}}$. The above formulas seem valid for the mechanism taking place in molecular sieve chromatography, which is particularly the case for the equation for low-molecular weight substances. Hjertén (18) derived, using a detailed thermodynamic treatment, the following equation for homologues: $-\log K = c_1 \times M + c_2 \times M^{\frac{2}{3}} + c_3$. The factors include the pressure difference of the system (c_1), the interfacial tension (c_2) and the activity coefficient (c_3). The importance of the swelling pressure, which corresponds thermodynamically to the pressure difference, has been reported for low molecular weight substances (19). For proteins the simplified formula $-\log K = a \times M^{\frac{2}{3}} + b$ often suffices to

describe the relation of K to M. The concept discussed above does not take into account the structure of the matrix (= the stationary phase). Examples follow in which the mechanism of molecular sieve chromatography is discussed with regard to the structural aspects involved.

1. The geometrical exclusion principle by Porath (20) and Squire (21).

2. The principle of restricted diffusion by Ackers and Steere (22), and Ackers (23).

3. The Ogston-Laurent gel model (24,25).

4. The geometrical and statistical pore size distribution model by Ackers (26).

Ackers' principle of restricted diffusion is an attempt to explain the hydrodynamic resistance to motion of the solute in the matrix by correlating Stokes' radius, r, of the protein and the effective pore-radius of the gel with the elution parameter. Laurent and Killander's structural exclusion model considers the network of the gel as rods of infinite length L and the radius R related to the fraction of the available volume. The formula $-\log K_{av} = L (r + R)^2$ has been found useful in both molecular weight determinations and for the estimation of the thickness of the polymer chain. A further development regarding the heterogenity of the gel extends the scope of this formula (27). A requirement for true molecular sieve chromatography is the existence of a homogeneous system, be it hydrophilic or hydrophobic, with the solvent and matrix showing the same degree of polarity as the solute. The relatively low Rf-values of DNP aspartic acid and naphthol green, obtained in the work discussed here, are not due to any molecular sieve action but are simply caused by sorption of the aromatic ring systems onto the gel matrix. This occurs if the polarity of solvent/matrix differs in relation to the solute. A Boltzmann-Brönsted equation may be used to describe the distribution of the solutes under conditions of linear concentration isotherms for both the above molecular sieve and sorption chromatography, as well as for "partition" chromatography. This would then represent a general concept of "distribution" chromatography.

Publication (II=3) describes the preparation of various gel materials, mostly acrylic polymers, applicable in separation. Ideally, on preparing matrices for separation, one should try to get matrices "tailor-made" for specific problems or applications.

In other words, besides optimizing "environmental" conditions such as using different solvents, the use of one specific matrix for one specific separation should be attempted. Polymers based on acrylic monomers are extremely useful for making a large variety of gels. They allow a great freedom of choice because gels can be made of:

a. varying porosity

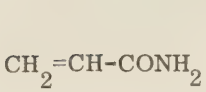
b. great chemical variation due to their ability to form different copolymers

c. easy chemical modifiability.

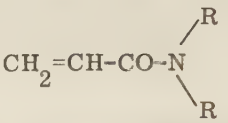
Furthermore, these matrices are excellent supports for the binding of ligands such as inhibitors or enzymes to be used in a new development in separation, that of affinity chromatography based on biospecific interaction (see Part B). Different preparations of porous material of cross-linked polymers for separation, with special reference to the molecular dimension of the substances, are described or proposed. These polymers are prepared from monomers containing C=C double bonds and cross-linking agents with at least two C=C double bonds to form copolymers. Copolymers can also be obtained on first preparing linear polymers, which subsequently are cross-linked with the below mentioned cross-linking agents or bifunctional compounds such as hydroxyl- and amino-reactive agents.

Some of the more important monomers which either have been or may be used in the preparation of polymers and which are mentioned (II=3) are the following: acrylamide, N-substituted acrylamide, methacrylamide, N-methacrylamide, acrylic acid, methacrylic acid and their respective esters, acrylonitrile and methacrylonitrile. Other monomers proposed are vinyl chloride, vinyl acetate, vinylmethylether, vinylethylether, styrene, butadiene, dimethylaminostyrene, and vinylsulfonic acid. Cross--linking agents listed are: N, N´methylene bis (acrylamide), N, N´methylene bis (methacrylamide) and derivatives; esters with polyvalent alcohols of acrylic, methacrylic and crotonic acids, acrylic acid anhydride, divinylbenzene, hydroxyl- and aminoreactive bifunctional compounds such as aldehydes (e.g. glyoxal), ketones, alkyl dihalogenides, aryldihalogenides, disulfohalogenides; and polyvalent acids. The formulas of the major monomers of acrylic type are given in figure 1.

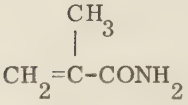
Figure 1 Major monomers and cross-linking agents cited in the text



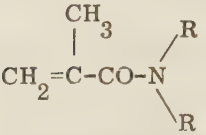
acrylamide



N-substituted acrylamide

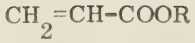


methacrylamide

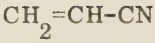


N-substituted methacrylamide

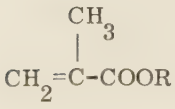
(R = CH_3, C_2H_5)



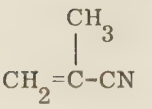
acrylic acid



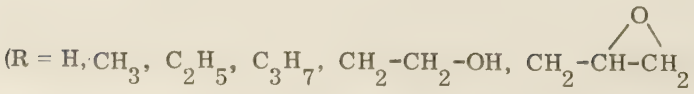
acrylonitrile



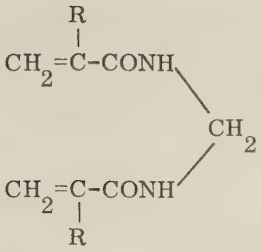
methacrylic acid



methacrylonitrile

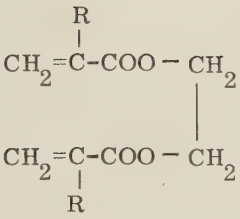


(R = H, CH_3, C_2H_5, C_3H_7, CH_2-CH_2-OH, CH_2-CH-CH_2)



N,N´methylene bis(acrylamide) (R = H)

N,N´methylene bis(methacrylamide) (R = CH_3)



ethylene glycol diacrylate (R = H)

ethylene glycol dimethacrylate (R = CH_3)

For the preparation of neutral hydrophilic polymers, monomers such as amides, sulfonamides and their derivatives, methyl acrylate and methyl methacrylates and their derivatives, as well as vinylmethylether, should be chosen. Besides the neutral hydrophilic and hydrophobic cross--linked polymers of different porosity, those of ionic nature are also of interest. To obtain ionic matrices three procedures may be used:

1. Simultaneous copolymerization of neutral monomers with ionomers.
2. Introduction of ionic groups on the preformed matrix.
3. Substitution of the linear polymer chains with ionic groups prior to their cross-linking.

Thus a broad variation of ionic matrices with different degrees of substitution may be obtained. Irregular distribution of the monomers may often be found when using the first procedure. With the second a gradient distribution between outer matrix and nucleus may occur. With the last, however, a more random distribution of ionic groups will be found. The above three techniques are also used in the manufacture of copolymers and modified polymers the products of which are distinguished by the nature and quantity of polar groups as well as their distribution along the polymer chain. For the separation of biological material, ionic matrices based on a hydrophilic backbone are of special interest. The separation principle then taking place includes both molecular sieving as well as sorption and ionic sorption (ion exchange).

The polymerization is carried out in a liquid medium as bulk or directly as bead polymerization (see also publication IV=6). This procedure in liquid medium has the effect that porous polymers of defined structure are obtained in contrast to polymerization in substance. The structure and thus the magnitude and distribution of porosity within the gel is dependent on the total concentration T, and the relative concentration of cross-linking agent C, as well as polymerization kinetics and other conditions. These parameters were first used (publication I=2) to permit characterization of the cross-linked polyacrylamide preparations applied. The influence of the amount of T and C of such polymers upon the resolution of substances has been investigated for cross-linked polyacrylamide (28). Increasing the total concentration of the monomers while keeping the concentration of the cross-linking agent constant, decreases the resolution of proteins. T thus determines the pore size of the gel. Conversely, C will also influence pore size. Thus it was found that polyacrylamide with C = 5 % yields the minimal average pore size (using constant total concentration). Above and below 5 %, pore size will be larger; such transition points are a characteristic of cross-linked polymers. Preparations with C-values above the transition point, again keeping T constant, turn more and more turbid indicating beginning micro-precipitation or organization leading to larger pores (29). There is a continuous transition from gel to so-called poroidines (capillar networks) of the polymer, finally going towards complete precipitation and phase separation. Another method of preparing porous bodies which have permanent porosity is the use of a solvent system in which the product cannot or only can swell to a limited degree. This technique of producing poroidines was applied to styrene divinylbenzene polymers by Moore in 1964 (30) with the aim of obtaining a matrix especially suited for the separation of high-molecular hydrophobic substances (gel permeation). Kun and Kunin (31) applied this principle in 1962 to the preparation of ion--exchangers based on macroreticular resins (a term these authors coined).

A third technique for making porous bodies is utilized in the sintering process in which polymeric particles are subjected to high pressure. Three main types of porous polymeric bodies or coherent-disperse systems of cross-linked polymers can be distinguished (32).

description		system of 1st order	structural elements
1.	gel	reticular	fibrillar
2.	spongoid	spongoidal	laminar
3.	poroidine	poroidinal	corpuscular

Gels (type 1) consist of crumbled and clustered structural elements; only an "ideal gel" has a regular lattice (27). A gel can also be described as an visco-elastic network with rigid elements. On the other hand, poroidines (type 3) consist of a rigid capillary network of corpuscular matter connected by mutual attachment. Various gradations from gel up to poroidine can be found. The matrices of gel and poroidine type described above can used in "molecular sieve" chromatography as well as in ion exchange, ion exclusion, "partition" and adsorption processes. Matrices of poroid type are also distinguished by their high capacity. The acrylic types are very valuable for separation and analytical distribution studies. Their excellent chemical resistance and inertness to biological degradation, particularly those composed of the methacrylic esters, es noteworthy.

To the polymers reactive groups can subsequently be introduced. Such activated groups can then react with various ligands and be applied for specific sorption (affinity) for separation, including chromatographic processes. Such sorption has been utilized in the separation of solubilized mitochondrial membrane proteins (33). The matrix used was a polyacrylic acid resin (Bio-Rex 70) to which aliphatic amines of increasing chainlength and increasing degree of unsaturation were bound using thionyl chloride. The lipoproteins bound are believed to interact through their apolar site with the introduced sidearms on the matrix. Bile acids present were almost totally excluded due to free carboxyl groups still available on the matrix. Elution of the bound lipoproteins was then effected using detergents and salts alternately. Chromatographic profiles were obtained separating different cytochrome proteins (cytochrome aa_3 and cytochrome b) from other proteins. The separation efficiency was best with capryl (8:0) and linoleyl (18:2) arms on the matrix, both believed

to be in a mobile state on the gel. Stearyl (18:0) substituted matrices were less efficient; here the lipophilic arms are believed to be in a sort of solid state, rigidly ordered. It is likely that such separations could be even more improved by using hydrophilic gels of acrylic types of higher porosity and "tailoring" of ionic charges on the matrix.

In Table 1 a survey of various matrices and different retarding forces leading to chromatographic separations is given. Comprehensive treatment of various aspects of separation using different kinds of gels are given in a number of reviews (34-40).

Various substances separated and the pertinent matrix utilized, are summarized in Table 2 (41-66). In all cases cross-linked polyacrylamide was used. The numbers given as 10^3 atomic units (Dalton) for the commercial preparations represent approximate exclusion limits. Conventional enzyme separations have not been included in the list. Examples of successful separations of immunoglobulins and their subunits, peptides, and glycoproteins are given. In the cases of peptide separation, their molecular weights have been determined as well (45). In studying the aggregation of 7S globulins this technique has been valuable in the analysis of their distribution pattern according to size (48). A technique of fractional precipitation, with the gel serving as carrier, has been applied in the example of purification of a complement fraction (49, 50). Even basic proteins and polyuridylic acid could be separated due to adsorption effects on part of the gel. Binding of substrates, inhibitors and drugs to enzymes has also been investigated. The fractionation of oligosaccharides according to size could be achieved from synthetic mixtures as well as from natural sources (53, 54). Quantitative evaluation could successfully be carried out. Nucleotides, nucleosides and corresponding bases were also used for chromatographic fractionation. Here sorptive interaction is involved in the separation, the strongest interaction taking place with nucleotides. The separation of nucleic acids is another important area. The behavior of inorganic salts on such matrices with the specific interaction of anions (65, 66) is of interest. In cases of sorption, the amide groups may exhibit acid-base behavior and show dipole effects. Other applications of these gels may be mentioned: exchange of buffers, desalting, concentration of polymers, basket centrifuge separation and application as an anticonvective medium in zone electrophoresis. Besides the usually applied column technique, thin-layer chromatography is also used, preferentially for application on a micro-scale.

Table 1. Matrices and Separation factors in chromatography

Matrix	Retarding Force	Dependence of Separation	Description
containing			
ionic groups	ionic force	ionic nature, polarity	ionic sorption, ionic exchange, sorption
dipole "	dipole interaction		
hydrogen-active groups	hydrogen bond		
non-polar groups	London dispersion forces		
activated "	covalent reaction	reactivity	reaction
support and/or part of liquid two-phase systems			"partition"
support for molecular specific ligands	complex formation	complex strength	"affinity" biospecific sorption specific sorption
gel structure	interfacial tension swelling pressure	molecular dimension	molecular sieve
poroidine structure	interfacial tension	molecular dimension	molecular sieve
molecular dimensional pores		molecular dimension	molecular inclusion
molecular specific holes		molecular configuration	molecular print

Table 2. Examples of different biological materials separated using cross-linked polyacrylamide

Matrix		Materials separated	References
Bio-Gel	P 200	IgG and IgM immunoglobulin	(41)
	P 150	papain-digested immunoglobulin	(42)
	P 60, P 100	Fc of IgG	(43)
	P 150	CNBr-peptides from rat skin collagen	(44)
	P 100	peptides	(45)
	P 30	glycoproteins	(46)
	P 2	desalting of peptides	(36)
	P 100	solubilized units of human erythrocyte membrane	(47)
	P 200	IgG (aggregation)	(48)
	P 10	complement	(49, 50)
	P 2	basic proteins from saliva	(51)
		and rattlesnake venoms	(52)
	P 2	oligosaccharides	(53, 54)
	P 6, P 10	polysaccharides	(55)
	P 2	nucleotides, nucleosides, bases and desalting	(56-59)
	P 2	nucleotides: ATP, ADP, 5-AMP and 3-AMP	(60)
	PA (T=5, C=5)	t-RNA from ribosomal RNA	(61)
	P 100	t-RNA	(62, 63)
	P 30	ribosome polyuridylic acid complex	(64)
	P 2	inorganic electrolytes	(65, 66)

PART B

Acrylic polymers as matrix for enzymes

Three principal methods have been established for binding or immobilizing enzymes to or within an insoluble matrix or support:

a. entrapment within a matrix

b. adsorption on a matrix

c. binding through covalent linkages to a matrix

Furthermore, enzyme aggregates have been obtained by intermolecular coupling using e.g. dialdehydes such as glutaraldehyde, leading to insoluble aggregates (67).

Many different matrices widely used are:

1. acrylic polymers
2. dextrans and agarose
3. cellulose
4. porous glass
5. nylon

In addition to these supports other special matrices have been employed. The recent use of living microorganisms as a matrix may be mentioned more as of a curiosity (68). The use of soluble matrices such as that of a recently used (69) amino-s-triazinyl derivative of dextran with the soluble α-amylase-polymer conjugate kept in an ultrafilter reactor may be considered as a sideline to the development of enzymes bound to insoluble matrices. Immobilized enzyme preparations have a number of applications in analysis; as enzyme electrodes (70) and in combination with flow microcalorimetry (71), in enzyme therapy and in enzyme technology, for example in the resolution of racemic mixtures of amino acids into their optical antipodes using bound aminoacylase (72), and in steroid transformation processes (73).

Since it is known that many enzymes are localized on or within membranous cell structures, such immobilized enzyme preparations constitute valuable model systems. The complexity of biological membranes makes the separation of the different parameters acting in vivo difficult, whereas with an artificial model system single microenvironmental parameters can be studied.

The practical and theoretical aspects of matrix-bound enzymes as well as their preparation have been discussed in some recent review articles (74-80). Special treatment of different aspects of the microenvironment around matrix-bound enzymes resembling those of natural membrane-bound enzymes is given in references (75, 79, 80).

In this part of the thesis, covering publications (III=4) and (IV=6), special attention will be given to the technique of entrapment of proteins within a gel-network (a) using acrylic polymer matrix (1).

Enzymes can be entrapped or included within a cross-linked gel matrix by carrying out the polymerization reaction in an aqueous solution containing the enzyme. The resulting gel bulk can be mechanically fragmented and sieved according to size. The most widely used polymer is that of acrylamide cross-linked with N, N´methylene bis(acrylamide). The first report on using an acrylic derivative appeared in 1963 (81). A polymer preparation obtained from N, N´methylene bis(acrylamide) and Al $NH_4(SO_4)_2$ was described with the enzymes kept immobilized within the complex formed. However, the use of a truly cross-linked polymer, that of polyacrylamides was reported in 1966 by the author (III=4) and independently by Hicks and Updike (5) and Wieland et al. (82) in the same year.

The entrapment technique of enzymes compares favorably with the other technique most frequently used, that of covalent binding of enzymes to a matrix because of the following two advantages. First, since no covalent linkages are involved, no derivatization of the enzyme takes place and the enzyme is thus likely to resemble its native state. Second, the gel network acts as a barrier and protects the enzyme against mechanical damage and enzymic degradation exerted by proteolytic enzymes likely to be present in incubation mixtures. The embedding of the molecules within the tight network, however, renders such preparations less suitable for the technique of affinity chromatography.

Publication (III=4) describes the technique of entrapping the enzymes trypsin and orsellinic acid decarboxylase. The activity of the immobilized decarboxylase was about 30 % of that of the free enzyme. This decreased activity may be caused by either partial inactivation of the enzyme and/or diffusion hindrance of substrate/product. In a thorough study to evaluate both parameters, Bernfeld et al. (83) entrapped radioactive aldolase. The ratio of enzyme activity to radioactivity in the aqueous phase remaining after the completion of the polymerization was about 25 % lower than the original soluble aldolase. This enzyme inactivation was interpreted to be due to the polymerization catalyst. Further, the ratio of enzyme activity to protein in the gel indicated that four times as much protein was present as indicated by the catalytic activity. To explain this it was suggested that only the aldolase at or near the surface of the gel particle is active (substrate saturation). In addition the authors showed that some part of the enzyme could be released by ultrasonically disrupting the gel particles. When solubilized this way, the aldolase was restored to activity. In a recent study by Degani and Miron (84) on the entrapment of acetylcholinesterase, the authors ascribed some of the decrease in enzymic activity observed to partial denaturation of the enzyme caused by the monomers. However, the evidence given in their statement was based on the decreased activity of the enzyme when assayed in solutions of acrylamide. Such a conclusion should be drawn with caution, since during the gel formation the monomer acrylamide is present only for a short period of time. It should be pointed out that some radical formation may take place leading to partial denaturation.

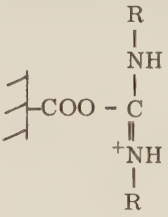
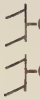
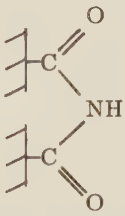
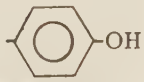
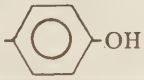
The first reported entrapment of intact microorganisms into a gel, namely that of lichens, is also described (III=4). Lichen columns prepared in this manner were used as a source of esterase and decarboxylase activity. The encagement of entire microorganisms will be advantageous in a number of situations, overcoming the necessity for laborious and difficult steps involved in enzyme preparations. Such preparations may also show greater stability. Moreover, the necessity of adding cofactors may be eliminated. Other examples are the entrapment of fungal cells containing 11 β-steroid hydroxylase, yielding preparations useful in steroid transformation processes (73), as well as that of cells from Streptococcus for the production of ornithine from arginine (85). The entrapping technique described has also been applied to multi-enzyme systems. The concomitant entrapping of two enzymes working in consecutive order, hexokinase and glucose-6-phosphate dehydrogenase, present in the same gel particles, has been described (86), as well as the entrapment of four enzymes of the glycolytic pathway in separate particles arranged in sequential order in column (87).

The application of acrylic polymers for use in the preparation of immobilized enzymes is not restricted to the entrapment technique. Acrylic polymers have been used extensively for the covalent attachment of proteins or ligands. This aspect will be trated in more detail in the next section, which deals with affinity chromatography or biospecific adsorption. Proteins have been attached to copolymers of acrylamide/ /acrylic acid using watersoluble carbodiimides (88), to copolymers of acrylamide/hydroxyethylmethacrylate (88) using the cyanogen bromide method (89), and to various derivatized polyacrylamide preparations (Table 3).

The principle of entrapment of enzymes within a gel-lattice is used in the preparation of enzyme electrodes. Here a membrane of acrylamide gel-immobilized enzyme is prepared by polymerization around an electrode. The enzyme electrode formed acts as a miniature chemical transducer which functions by combining an electrochemical procedure with immobilized enzyme activity. Glucose has been determined by using glucose oxidase entrapped around a polarographic oxygen electrode (90), and urea by using urease immobilized around a cationic glass electrode sensitive to ammonium ions (70).

The method of bulk polymerization followed by physical fragmentation (previously discussed) has the disadvantage of giving non-homogeneous preparations with some enzyme leakage occurring at the ruptured surfaces. Polymer beads of uniform size containing the entrapped active enzyme can be obtained by applying the conventional bead-polymerization process (publication IV=6). The general principle consists of dispersing the water solution containing enzyme and acrylic monomers in a hydrophobic phase, after which polymerization is initiated. The average bead diameter can be reduced from on the average 100-250 to 10 μm by changing polymerization conditions. Mainly the enzyme trypsin has been studied and gives preparations with as much as 70 % residual activity. The same enzyme has been used in a combination of the above general encagement technique and that of covalent binding, and gives preparations in which the enzyme is kept entrapped and covalently bound within the matrix. This may be of value in entrapping biological material of low molecular weight such as trypsin which might otherwise escape to some degree through the pores of the gel. Such preparations could be obtained easily on copolymerizing the two monomers acrylamide and acrylic acid together with the cross--linking agent N,N´methylene bis(acrylamide) in the presence of both enzyme and a water soluble carbodiimide. The general applicability of the bead-polymerization technique was demonstrated using more labile enzymes such as lactase F, a fungal lactase. These preparations show

Table 3. Most widely used procedures leading to covalent binding of proteins or ligands to acrylic polymers

original matrix	"coupling" agent	intermediate matrix	major reacting groups of protein or ligand
	carbodiimide		- NH_2 - SH
	BrCN		- NH_2
	$H_2N \cdot NH_2$		- NH_2 - COOH
	$H_2N(CH_2)_nNH_2$		- " -
	HNO_2		- NH_2 - SH 
	$OHC(CH_2)_nCHO$		- NH_2 

* Requiring additional "coupling" agent.

a high residual enzymic activity. Another labile enzyme, the copper--containing fungal laccase (91-93), was entrapped by this procedure. Active preparations (oxidizing approximately 100 μmoles of catechol/min per g of dry polymer) were obtained on entrapping laccase (to be published).

Preparations somewhat resembling the "enzyme-beads" described above are obtained by using the microencapsulation technique. A free solution of enzyme is kept enclosed within a thin semipermeable membrane such as nylon or collodion (94). A third variation is the recently described entrapment of enzymes in liposomes (95), in which a series of concentric bilayers of phospholipids alternates with aqueous compartments containing the entrapped enzyme. Of the three different types of preparations the one involving entrapment of enzymes in acrylic polymers appears to give the best model system for enzymes in vivo embedded in membranous structures or gel-like surroundings. Microencapsulated enzymes, on the other hand, make poor models since they are kept within a sack but in free solution. Liposomes also do not represent good models, as here most of the enzyme activity is latent and expressed only after destruction of the liposomal membrane structure. All three preparations have great potential in the treatment of enzyme deficiency diseases, since undesirable immunological responses may be prevented. The two preparations mentioned first can be used in an extra-corporal shunt but also, though to a lesser degree, in introduction directly into the bloodstream. The liposomal preparations may be useful as enzyme carriers directing the enzyme to a particular tissue. In the treatment of kidney failure, both microencapsulated or acryl-entrapped enzymes may find use in the removal of urea. In this context preparations described in publication IV-6 appear to be superior due to their mechanical stability and uniform structure. This stability, as well as inertness to microbial attack, makes them seemingly ideal matrices in the developing area of enzyme technology.

The technique of affinity chromatography or biospecific adsorption has received a considerable amount of interest during the last few years, as illustrated by a number of recent reviews (96, 97). This technique is based on the unique biological property of many proteins or polypeptides to bind ligands specifically and reversibly and utilizes these specific biological functional properties in the purification of proteins for example. This is unlike conventional procedures such as gel chromatography, which are based on physicochemical criteria.

Affinity chromatography or biospecific adsorption can be applied to a number of macromolecular ligand systems. Specific adsorbents have been prepared to purify enzymes, various proteins such as vitamin binding and repressor proteins, antigens and antibodies, nucleic acids, drug or hor-

mone receptors, peptides formed in organic synthesis, as well as intact cell populations. The different matrices most frequently used for the attachment of ligands or proteins are: polystyrene, cellulose, glass, agarose and polyacrylamide derivatives. Of these, the latter two have been found superior due to the large porosity of these polymers, which is important in affinity chromatography. Since in a number of ligand--complex systems, the dissociation constant is 10^{-4} M or even greater, a high concentration of readily available ligands is necessary and will permit many interactions, thus retarding the downward migration of the proteins within the column. Like the beaded derivatives of agarose, the synthetic polyacrylamides are highly useful in affinity chromatography. They have uniform physical properties and porosity. The fact that the latter have not yet found as wide use as agarose preparations in affinity chromatography may be due in part to their somewhat lesser porosity. The polyethylene backbone gives them physical and chemical stability. Two principal binding methods for ligand or protein have been applied to polyacrylamide matrices and used in affinity chromatography:

a. entrapment

b. covalent binding.

Using the entrapment technique, DNA has been included within polyacrylamide particles. DNA polymerase from Escherichia coli was then purified 200-fold on a single passage through the column (98). Such preparations show great potential for hybridization experiments.

For technique b. the polycrylamide gels have to be derivatized first; in addition, introduction of a "spacer" molecule between ligand and matrix has been useful in a number of cases. Polyacrylamide beads can be successfully derivatized on reaction with glutaraldehyde as recently illustrated (99, 100). It is believed that one aldehyde group reacts with the amide, leaving one aldehyde group free for coupling with protein. This interpretation is in accord with previous findings that cross-linked polyacrylamide gels can be prepared by substituting N, N´methylene bis(acrylamide) with dialdehydes such as glyoxal (II=3). The other procedure of polyacrylamide derivatization, based on the work of Inman and Dintzis (101), involves conversion of the carboxamide side group to hydrazide groups followed by conversion into acyl azide derivatives with nitrous acid.

With such preparations, some enzymes have been purified by the principle of affinity chromatography. In one study, staphylococcal nuclease was

purified using the bound competitive inhibitor, pdTp-aminophenol (102). In this context it was shown that insertion of a spacer, the tripeptide glycylglycyltyrosine, increased the capacity of the column material (Bio-Gel P-300) at least 5-fold compared to direct binding of the inhibitor (Fig 2). This was based on a previous study where the importance of such anchoring arms was shown (103). In this study it was found that no binding at all of α-chymotrypsin to the inhibitor D-tryptophan methylester occured when coupled directly to Sepharose. However, on first inserting a spacing group, ϵ-aminocaproyl, strong binding of the enzyme took place.

The purification of dihydrofolat-reductase by using 4-amino-10-methyl--pteroyl glutamic acid bound to aminoethyl-Bio-Gel P-150 (104) has been reported.

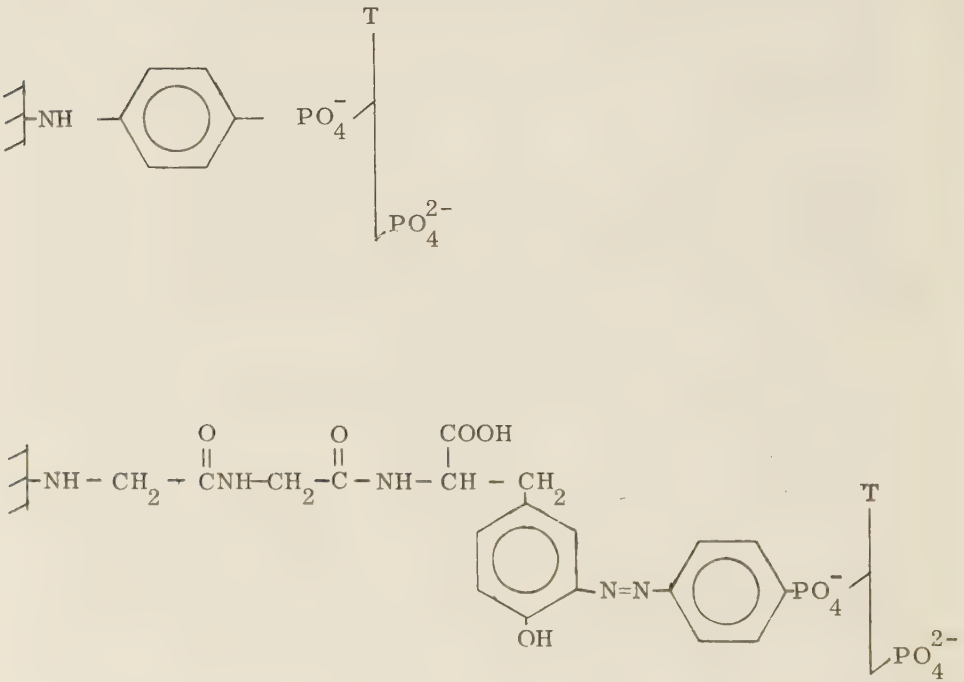


Figure 2

The application of affinity chromatography to the purification of antigens or antibodies, does not require as much sophistication as does the isolation of enzymes. This is due in part to the fact that dissociation constants of antibody-antigen interactions are generally smaller than 10^{-7}M, indicating stronger binding. Further introduction of spacer molecules does not appear necessary because for example in the case of globular protein antigens, enough antigenic determinants will be located at some distance from the matrix to be sterically accessible to the antibody. This may, in part, explain the successful application of "older" matrices such as cellulose for the preparation of immunoadsorbents (74). Nevertheless, the newer gels, including polyacrylamide, find increased application in this area due to their non-antigenic characteristics. Again, the entrapment technique or covalent binding has been used. In one study antibodies of the antigen angiotensin (mol.wt. 1000) were entrapped in polyacrylamide (105). The molecular weight of the antibodies, IgG, is 160,000 and can thus be entrapped. These preparations were then used as solid phase reagent for radioimmunoassays using ^{125}I labelled angiotensin.

Another paper reports entrapping of antigens for the isolation of antibodies (106). To obtain a gel of high porosity necessary for accessibility of the large antibodies to the entrapped antigen, in this case haemoglobin and IgG, a change in T-C (see nomenclature in part A), that is total monomer concentration as well as the ratio acrylamide/N,N´methylene bis(acrylamide), had to be made. Using the resulting macroporous matrix, the corresponding antibodies could be selectively bound and subsequently eluted off. Subsequent studies by the same authors led to the development of polyacrylamide-entrapped immunoadsorbents of high binding capacity for either antigen or antibodies (107). Such preparations offer the additional advantage of being storable for months and may be used repeatedly without loss of binding capacity.

An interesting development in the immunological field has recently been reported in the isolation of special cell populations using an azo-coupled phenyl-β-lactoside (lac) antigen attached to polyacrylamide beads (108). Cell populations that produce antihapten antibodies of corresponding specificity could be isolated using such preparations as affinity columns. In another study with these azophenyl-β-lactoside polyacrylamide preparations, lymphocytes that specifically bind a lactoside hapten were isolated from the spleens of unimmunized mice (109). Such preparations also show potential for specifically purifying cell populations with characteristic membrane-receptor proteins.

Besides isolation of antigens, antibodies or cell populations, beaded polyacrylamide gels may find application as an aid in a recently developed quantitative evaluation method for antibodies. Here the amount of agglutinate is measured photometrically as coating on an inclined surface in a cuvette (110-112). The agglutinates are aggregates of erythrocytes containing bound antigens to which antibodies are linked. Replacement of the erythrocytes with an immunologically inert matrix, such as polyacrylamide beads, offers a definite advantage (113).

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